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Natural entrainment of circadian systems

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CHAPTER 1

Background and thesis overview

INTRODUCTION

Life has evolved on a rotating planet under continuous exposure to the cycle of day and night. During evolution organisms have formed an internal representation of the external daily cycle, and the endogenous generation of circadian (~24 h) oscillations has become firmly rooted in the DNA of virtually all organisms. These internal circadian oscillations, or biological clocks, drive daily patterns in numerous behavioural and physiological processes. By virtue of being synchronized to the environmental light-dark cycle, the circadian system allows organisms to optimize the timing of their daily routines, such as in feeding and sleeping. Also the timing of annual cycles in, for example, reproduction or migration, depends on the circadian system.

In complex organisms specialized groups of cells (pacemakers) in the central nervous system generate the circadian oscillation and impose this on the rest of the organism. In general, such circadian pacemaking systems are synchronized by the light-dark (LD) cycle; a process called entrainment. The mechanism of the entrainment process has been intensively studied and became well understood by the mid-70's. Laboratory studies have shown extensively that the circadian pacemaker in animals responds to brief light pulses by shifting its phase. Theoretical work, partly based upon these empirical data, resulted in testable models predicting how circadian oscillators are synchronized to environmental daily cycles (Pittendrigh and Daan 1976b). The theory was only tested in the laboratory, mostly using square wave LD cycles. The validity of the theory for entrainment under natural conditions has received almost no attention. Nonetheless, this theory has become widely accepted.

Entrainment studies in mammals are almost solely based upon laboratory measurements in nocturnal rodents. However, they are also applied to predict natural behaviour in diurnal mammals (including humans). Studies in diurnal rodents would probably form a more reliable basis to predict human circadian entrainment, but there are few empirical studies available.

This thesis was inspired by the need for data on natural activity patterns in a diurnal mammal, and the need to test whether theories on entrainment hold under natural circumstances. Detailed descriptions of behaviour, light conditions, and light perception, were used to analyse natural circadian entrainment in the European ground squirrel (*Spermophilus citellus*). This descriptive work was complemented with laboratory experiments and theoretical modelling to quantify causal relationships and obtain insight in the complex system of natural entrainment.

The European ground squirrel is a burrow dwelling rodent that is active above ground during most of the light phase of the day. It exposes itself to large variations in light intensity by moving in and out of its burrow during the active phase. Behavioural influences on the light perceived in nature are extreme in diurnal burrowers. Such behaviourally induced variations in light intensity are a challenge to the available theoretical entrainment models. Understanding the responses of the circadian system to these large light intensity variations may also gain better insight in entrainment mechanisms of the human circadian system, since our own behaviour also strongly influences the light environment to which we are exposed. As a result, this study may lead to a better understanding of circadian entrainment for a variety of species with different natural activity patterns.

The mammalian circadian pacemaker

The mammalian circadian pacemaker is located in the suprachiasmatic nuclei (SCN). The brain area containing the SCN is located at the ventral margin of the hypothalamus, on top of the optic chiasm and flanking the ventral tip of the third ventricle. The hypothalamus is considered to be an evolutionary ancient brain area, shared among all higher vertebrates. The function of the SCN was first presumed on the basis of precise local lesions that abolished circadian rhythms in the rat (Moore and Eichler 1972; Stephan and Zucker 1972). Definitive proof has later been provided by transplantation experiments: SCN-lesioned, arrhythmic hamsters developed circadian rhythmicity when receiving an SCN tissue graft and, more importantly, they displayed the period of the donor (Ralph et al. 1990). This proved beyond any doubt a property of this specific brain area: overt behavioural circadian rhythms are driven by the SCN and their periods are a property of the SCN.

The SCN is sensitive to light, as shown by neuroanatomical, neurophysiological, and behavioural data. The SCN in mammals is found at the location where the optic nerves enter the brain. In all mammals studied so far, including ground squirrels (Smale et al. 1991), projections from the retina to the SCN (retino-hypothalamic tract, RHT) have been identified (Meijer and Rietveld 1989; Card and Moore 1991). The retinal photoreceptors projecting to the SCN, although not yet identified, seem to be part of a well preserved and ancient mechanism (Roenneberg and Foster 1997). The SCN show strong immediate early gene expression (c-fos) in the SCN in response to a light pulse (Aronin ad al. 1990; Rusak ad al. 1990). Transcription of the mPer1 gene, involved in the molecular mechanism of the circadian clock in SCN neurons, is affected by light (for review see: King and Takahashi 2000). These responses are maximal during the subjective night, when also phase shifts of circadian activity rhythms following a light pulse are maximal (Johnson 1990; Beersma *ad al*. 1999). Ocular light perception is necessary for circadian entrainment by light (Richter 1967). In an experiment with ground squirrels, bi-lateral enucleation resulted in free-running circadian activity patterns even when the animals were kept under natural conditions outdoors (Nelson and Zucker 1981). This experiment showed that environmental zeitgebers other than light, such as the natural daily temperature cycle, have only marginal effects on circadian entrainment.

Intrinsic period: τ

Endogenous circadian oscillators have an intrinsic period (τ) that is close to 24 h. τ can be measured when biological organisms are deprived of an LD cycle, and any other entraining stimulus (*Zeitgeber*). Aschoff (1960; 1979) described systematic changes of the intrinsic value of τ with light intensity under continuous conditions ("Aschoff's rule"). Although τ can be very stable under such conditions, several experimental procedures have shown that it can also be altered for many cycles before it returns to its intrinsic value. The period (T) and photoperiod (proportion of light) of a 24-h LD cycle affect the endogenous period of the circadian oscillating system under subsequent free-running conditions ("*after effects*"; Pittendrigh 1960; Pittendrigh and Daan 1976a). Even a single brief light pulse induces τ changes that depend on the phase of the circadian system. They appear to have the same direction as phase shifts. These experiments indicate that τ has not a fixed value, but is a parameter of the endogenous pacemaker that is adjusted to conditions during entrainment.

Entrainment

The process of entrainment is the main topic studied in this thesis. Since the intrinsic period of a circadian oscillator may deviate from 24 hours, the circadian system must be accurately adjusted entrained to a daily environmental cycle of 24 hours. Entrainment can theoretically be obtained through daily adjustments of phase ($\Delta\phi$), or adjustments of period ($\Delta\tau$). In both cases, phase dependent sensitivity of the circadian system is required for entrainment. Although temperature cycles, feeding schedules, or handling all have been reported to affect the mammalian circadian system to some extent, light cycles surely are the strongest zeitgeber in most mammals (Richter 1967).

Models of entrainment

In the 1960's and 1970's the main theory on entrainment of living organisms by the LD cycle was developed primarily by Colin Pittendrigh [*e.g.* (1960; 1976b; 1993)]. This model was based upon the observation that the phase (ϕ) of a circadian rhythm can be shifted by a single brief light pulse. The size and direction of this phase shift ($\Delta\phi$) depends on the phase at which the circadian system is exposed to the light pulse: $\Delta\phi$ is a function of ϕ [$\Delta\phi(\phi)$]. Empirical data, collected in many species, showed that the circadian rhythm advances when a light pulse is given in the late subjective night or early subjective day. Delays of the rhythm are found when the light pulse is given during the late subjective day or early subjective night. When plotted against the phase of the circadian system, these phase shifts result in a phase response curve (PRC; Figure 1.1).

If the same brief pulse is applied periodically with a period close to 24 hours, the circadian system will adjust its phase on a daily basis resulting in entrainment of the circadian rhythm. When the duration of the light pulse is extended, resulting in a 24 h LD cycle, the subjective day of the circadian rhythm remains in phase with this LD cycle and the subjective night with the dark phase. The difference between the intrinsic period (τ) and the period of the Zeitgeber (T) needs to be corrected by these phase shifts on a daily basis to result in entrainment ($\Delta\phi(\phi) = \tau - T$). The amplitude of the PRC determines the so called *range of entrainment*: the range of T-values to which the oscillator can entrain. The decreasing

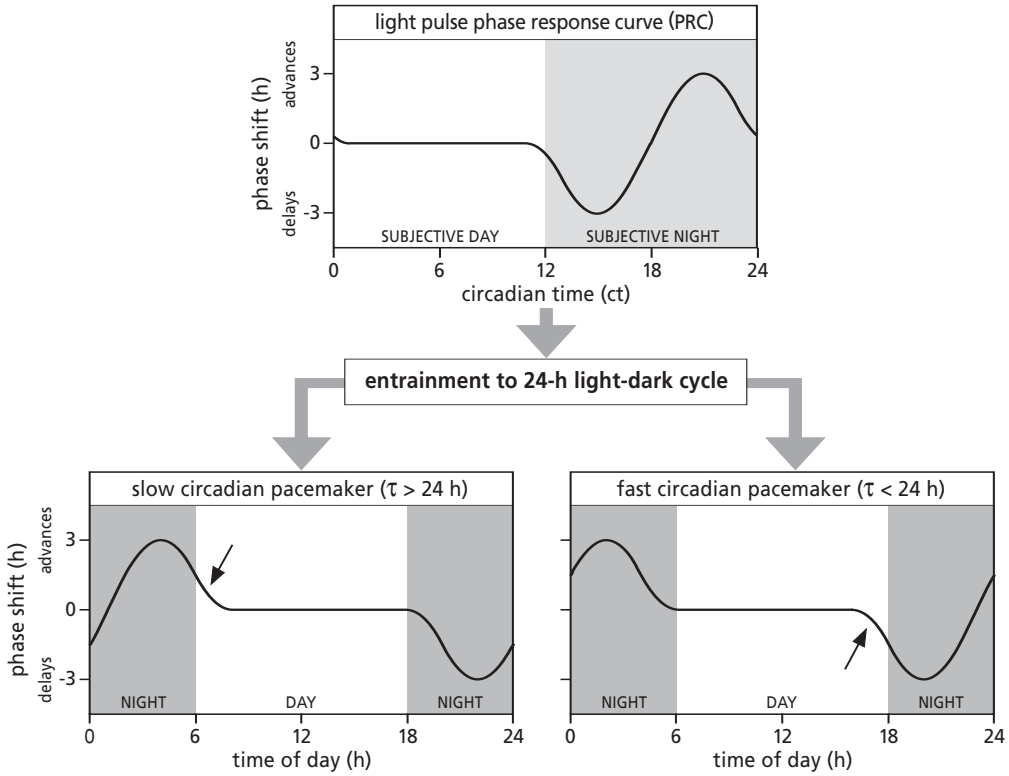

Figure 1.1 Illustration of the principle of entrainment by daily phase shifts. Top panel represents a phase response curve as can be found by applying single brief light pulses to a free-running animal against a background of continuous dim light. A light pulse perceived during the early subjective night will delay the free-running rhythm. When the light pulse is perceived during the late subjective night it will advance the free-running rhythm. Under a 24-h light-dark cycle (T), these phase shifts can correct the deviation of the intrinsic circadian period (τ) from 24-h. When $\tau > 24$ h, the subjective day will drift backward through the light phase of the day until the advance portion of the PRC (late subjective night) is illuminated to such an extend that daily advances correct for the difference between τ and T (lower left panel). When $\tau < 24$ h, the subjective day will drift forward through the light phase of the day until the delay portion of the PRC (early subjective night) is illuminated to such an extend that daily delays correct for the difference between τ and T (lower right panel).

negative slope of the PRC around the early subjective day and the increasing negative slope around the late subjective day yields a certain phase at which the phase shifts are exactly equal to the difference between τ and T. This results in a stable *phase angle difference* (ψ) between the intrinsic oscillator and the environmental light dark cycle. The model predicts that ψ will depend on the light intensity of the LD cycle, the difference between τ and T, and the (local) shape of the PRC. Figure 1.1 summarizes the principle of Pittendrigh's entrainment model. The upper panel shows the classical phase response curve with relative insensitivity during the subjective day, phase delays in the early subjective night and phase advances in the late subjective night. The lower left panel depicts the phase position of a

slow pacemaker. It tends to lag behind the daily LD cycle. Thereby the advance region of the PRC tends to drift in to the morning light which then elicits an advancing response correcting for the difference between t and T. In contrast (lower right panel) a fast oscillator drifts forward in time and is corrected by a phase delay elicited by light around dusk.

Although sufficient for explaining entrainment and phase angles during entrainment (in nocturnal rodents), the phase response model can not explain all experimental results. Some of the entrainment phenomena suggest that τ and PRC are not fixed properties of circadian systems, as would be required to quantitatively predict entrainment patterns, but are themselves subject to dynamic change (Pittendrigh and Daan 1976b). A different theoretical approach has emphasized continuous changes in the angular velocity of oscillators under the influence of light (Aschoff 1964; Wever 1966). In this approach, the assumption is made that part of the endogenous cycle is accelerated while another part is being decelerated during exposure to light. This is the principle of "*parametric entrainment*", as opposed to the "*nonparametric entrainment*" by instantaneous phase shifts in response to brief light pulses. Perhaps due to the fact that parametric entrainment less easily yields quantitative predictions the model has attracted much less attention. In contrast, Pittendrigh's phase response model for nonparametric entrainment has found wide acceptance among chronobiologists and has formed the basis for most of the work done in behavioural, physiological, and theoretical chronobiology.

The translation from models, based upon laboratory results, to the natural situation is not as straightforward as it may seem. First, day-night light intensity cycles in nature do not have a square wave shape, but have gradual increases and decreases around dawn and dusk. Second, clouds and other sources of shade introduce fluctuations in light intensity levels. Third, the behaviour of the animal itself may introduce large variation in the perceived light signal via positional changes, eye closure, and more important, retreat into burrows or other dark dwellings.

The European ground squirrel

The European ground squirrel or Suslik is a member of the *Sciuridae*. The species was referred to originally as *Citellus citellus* (Linnaeus 1766) and more recently as *Spermophilus citellus*. The distribution of the European ground squirrel is limited to open non-agricultural vegetation in middle Europe varying from the open 'puszta' planes to grassy slopes of low mountain areas. Ground squirrels have a pronounced endogenous annual cycle, in which hibernation during autumn and winter is followed by reproduction (one litter) in early spring. The summer season is used for fattening in preparation for the following 6-7 months of hibernation, starting at the end of the summer (Millesi *et al*. 1999b). As a result, body mass shows a pronounced annual cycle and varies, when calculated as a population average, between 280 and 400 g in adult males, and 200 and 300 g in adult females (Millesi *et al*. 1999b).

Because of its potential as an example species for quantifying natural daily and annual patterns in behaviour and energetics, a long-term project on the European ground squirrel was initiated in 1989 by D. Masman and S. Daan in collaboration with E. Millesi and J.P. Dittami (Department of Ethology, University of Vienna, Austria). In this framework, A.M. Strijkstra introduced the European ground squirrel to the Zoological Laboratory, in Haren

(University of Groningen, The Netherlands) for his PhD project on causes and consequences of hibernation patterns (Strijkstra 1999). The animals thrive well under controlled conditions in the laboratory and we managed to breed them in outside enclosures. The open habitat and diurnal activity of the European ground squirrel made it possible to study this species in nature. Collaborations between our group and the Ethology departments of the University of Vienna (E. Millesi) and the Eötvös University, Budapest (V. Altbäcker) made it possible to collect valuable data on activity patterns in the field.

Thesis overview

At an early stage in this research, L.G. Everts, A.M. Strijkstra, I.E. Hoffmann, S. Huber and E. Millesi collected full-day time budgets for European ground squirrels in a population near Vienna (Langenzersdorf), Austria. A complete documentation of observed above ground activity patterns over the entire activity season was obtained in two years (**Chapter 2**). It showed that the ground squirrels' above ground activity started long after dawn, and ended long before dusk. This finding seemed to be at variance with the classical theory of entrainment (see above) that assigns a crucial role to the twilight transitions. Although carefully executed, this study did not record the behaviour of the animals inside their burrows. The ground squirrels might have remained in their burrow entrances and observed dawn and dusk unnoticed by the observers. For a detailed description of the problem of natural entrainment in this species it was necessary to measure the self-selected light conditions. Using light-sensitive radio collar transmitters we were able to assess the timing of light perceived by the European ground squirrels in enclosures located at the Zoological Laboratory in Haren, The Netherlands, and at a field site at Bugac Puszta, Hungary (**Chapter 3**). This study confirmed that the ground squirrels do not observe the twilights from inside their burrows. This conclusion was corroborated by endoscopic observations in ground squirrel burrows at Bugac Puszta, Hungary, that revealed that the squirrels completely block the entrance tunnels of their burrows when retreating below ground in the afternoon (**Chapter 4**).

In addition to excluding themselves from external information on time of day during dawn and dusk on a daily basis, the ground squirrels also exclude themselves from such information during a large part of the year when they are hibernating in closed burrows. After hibernation the ground squirrels need to re-entrain to the environmental daily cycles. For practical reasons it was not possible to measure this process of re-entrainment by light sensitive telemetry as described in **Chapter 3**. As an alternative we implanted ground squirrels with small body temperature loggers. Body temperature is also driven by the circadian system and forms an excellent marker for internal circadian rhythmicity. Body temperature patterns before and during hibernation, as well as the process of post-hibernation re-entrainment is described in **Chapter 5**. A detailed follow-up study in the laboratory showed possible effects of hibernation on the circadian pacemaker and the effects of light on the circadian system after hibernation (**Chapter 6**).

If the twilight transitions are excluded as the primary entraining agent, alternative potent zeitgebers need to be considered. The spectral composition of sunlight has been suggested to be important for entrainment (Krüll *et al*. 1985; Roenneberg and Foster 1997). This hypothesis is mainly based on correlations between the solar spectrum and activity

patterns; the available experimental data are not convincing. We obtained detailed measurements of spectral composition of sunlight over the course of the day (**Chapter 7**). These showed that high levels ultraviolet (UV) light correspond closely with the above ground activity of the ground squirrels. Although the eye lens of diurnal mammals is known to absorb UV light, possible fluorescence mechanisms may enable the retina to detect UV light (Merker 1934; Jacobs 1992). We conducted a laboratory experiment in which the possible entraining effect of a UV light cycle was studied in ground squirrels and compared with nocturnal hamsters. In the latter species UV light is known to produce phase shifts of the circadian system (Von Schantz *et al*. 1997). It led to the conclusion that the role of UV light for natural circadian entrainment in diurnal, but also in nocturnal mammals is probably limited (**Chapter 7**).

In addition to photic stimuli, also nonphotic stimuli may have an effect on the circadian system. Nonphotic stimuli have been shown to entrain nocturnal mammals (Mrosovsky *et al*. 1989) and diurnal birds (Reebs 1989). The PRC for a variety of nonphotic stimuli in hamsters has a different shape than their photic phase response curve. As a result, phase angle differences during nonphotic entrainment are different from those found during photic entrainment. In contrast, in diurnal birds phase angle differences during nonphotic entrainment are similar to phase angle differences to a photic cycle. This is corroborated by a nonphotic PRC measured in the house sparrow (Reebs 1989), the shape of which is similar to a photic PRC. These differences may relate to differences between birds vs. mammals, or diurnal vs. nocturnal species, or social vs. non-siciaal species. Measuring phase angle differences during nonphotic entrainment in a diurnal mammal could add an important argument in the ongoing debate over the functional role of nonphotic entrainment. This issue has been experimentally addressed in **Chapter 8**.

The strictly diurnal above ground activity pattern and the absence of dawn and dusk perception in the European ground squirrel led to a new and integrative model for entrainment, involving both phase shifts and period changes of the circadian system (**Chapter 9**). This model contains elements of both the nonparametric (Pittendrigh) and the parametric (Aschoff) entrainment models. It is the combination of both models that has probably more explanatory power and may eventually lead to a simulation of the natural entrainment patterns of the European ground squirrel. The behaviour of this model under fluctuating light conditions is first described in general (**Chapter 9**), and later specified for the European ground squirrel (**Chapter 10**). The response of the model to a given LD cycle is fully dependent on the shape of the PRC and τRC. Both PRC and τRC were measured in the European ground squirrel and their relative contribution to optimal stability and phase angle difference is estimated. These measurements and simulations suggest an important role for τ (velocity) responses in natural entrainment, but did not prove this. The model proposed generates several testable predictions and its validity, assumptions and limitations need to be evaluated for a variety of circadian systems.